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Immunochemical Assays in Pesticide Analysis

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Immunochemical assays (immunoassays, IAs) are biochemical assays which work according to the law of mass action. They are based on the recognition of an antigen (Ag) or a hapten by antibodies (Abs). Abs are serum glycoproteins of the immunoglobulin (Ig) class and are produced by the vertebrate immune system against foreign material of high molecular mass. The result of the binding reaction between the Ab and an analyte is usually made visible by means of enzymatic, chemiluminescent, fluorescent or radioactive markers. According to the label used IAs can be classified into enzyme immunoassays (EIAs), radioimmunoassays (RIAs), fluorescence immunoassays (FIAs) or chemiluminescent immunoassays (CLIAs). The measuring range of most IAs for pesticides is in the parts

per trillion to lower parts per billion range. A lot of samples can be analyzed within a short time, while only low sample volumes are necessary. In many cases (water, some liquid food samples) no extraction step and no cleanup are necessary. Not all assays are completely specific to one single compound. Cross-reactivities of the Abs with haptens similar to the analyte can be observed. In some cases, matrix effects may occur, especially with soil or colored food extracts. Therefore, validation of the assays for the matrix of interest should be carried out. As IAs are usually targeted at a single analyte or a group of analytes, multi-analyte approaches using Ab arrays or a combination of immunochemical techniques with liquid chromatography (LC) are pursued.

1 INTRODUCTION

Interest in immunochemical assays for the determination of pesticides has been steadily increasing. IAs are now commonly applied for the analysis of contaminants in water, soil, food and body fluids.^(1–9) The first immunological experiments had already been carried out as early as the late eighteenth century when Edward Jenner, an English physician, used cowpox to prevent infection with smallpox. Based on these studies Louis Pasteur developed the use of attenuated strains of microorganisms for successful vaccinations. Emile Roux and Alexandre Yersin then found that immunity is caused by soluble compounds of microorganisms, which they called toxins. These toxins induce specific compounds in the immunized animal, which were named “antitoxins” by Emil von Behring and Shibasaburo Kitasato (1890) and are now called Abs. The Ab “generating” compounds are known as Ags.

Around the turn of the century it was shown that Abs are not only produced against microorganisms and their toxins, but also by other substances such as milk, protein or plant-derived toxins. Paul Ehrlich was the first to carry out quantitative studies on Ag–Ab interactions. The great interest in this field led to the first book on immunochemistry, published by Svante Arrhenius in 1907.⁽¹⁰⁾ Karl Landsteiner also belongs to the pioneers in immunochemistry. He systematically used small artificial molecules, which he called haptens, coupled to a carrier molecule for immunization. In 1923 Heidelberger and co-workers found polysaccharides to be antigenic as well.

Studies by Rodney Porter (1959) and Gerald Edelman (1961) have provided the chemical structure of the Ab molecule. The enormous variety of Abs was explained by Frank McFarlane Burnet in 1957, based on a hypothesis of Niels Jerne dating from 1955, the now widely accepted clonal-selection theory. It describes each Ab-producing

cell as carrying on its surface only one type of Ab as a receptor. The binding of a respective Ag to this receptor leads to a clonal expansion of this cell and to the maturation of Ab-producing cells.

Immunochemical methods have their origin in the medical field. The first IA, a RIA for the quantification of insulin in serum, was described by Yalow and Berson.⁽¹¹⁾ Later, radiolabels were replaced by enzymes in EIAs by Engvall and Perlmann⁽¹²⁾ and Van Weeman and Schuurs.⁽¹³⁾ Since then radiolabels have obtained broad application in medical diagnostics and environmental analysis.

IAs belong to the most common methodology in the field of immunoanalysis. Even though Abs are (still) produced by a biological process, IAs are nevertheless chemical analytical procedures. The basic principle applying to all immunoreactions is based upon the law of mass action. In the equilibrium reaction between a free Ag or a hapten, such as a pesticide, and the Ab forming the hapten–Ab complex HAb (=bound hapten), represented by Equation (1),



the affinity constant K determines the concentration ratio between the bound hapten and the free reaction partners, Equation (2):

$$K = \frac{[HAb]}{[H][Ab]} \text{mol}^{-1} \quad (2)$$

A low detection limit (DL) in an IA therefore requires a high affinity of the Ab toward the analyte, which is expressed by a high affinity constant. For further details refer to Hock et al.⁽¹⁴⁾

IAs are based upon the measurement of Ab binding-site occupancy by the analyte (Figure 1). This reflects the analyte concentration in the sample. Since the binding reaction does not produce a signal which can be detected by simple means, various markers, e.g. radioactivity, enzymes, or fluorescence, are employed for the detection of the immunoreaction (see section 3.2). However, more sophisticated techniques like some immunosensing methods do not rely on a label (see section 6).

2 ANTIBODIES

2.1 Antibody Structure

Immunochemical analysis is based upon the specific reaction between an Ab and its corresponding Ag or hapten. Abs are part of the vertebrate defense system (for more details refer to immunology textbooks, for example Golub⁽¹⁵⁾ and Roitt⁽¹⁶⁾). They are serum glycoproteins

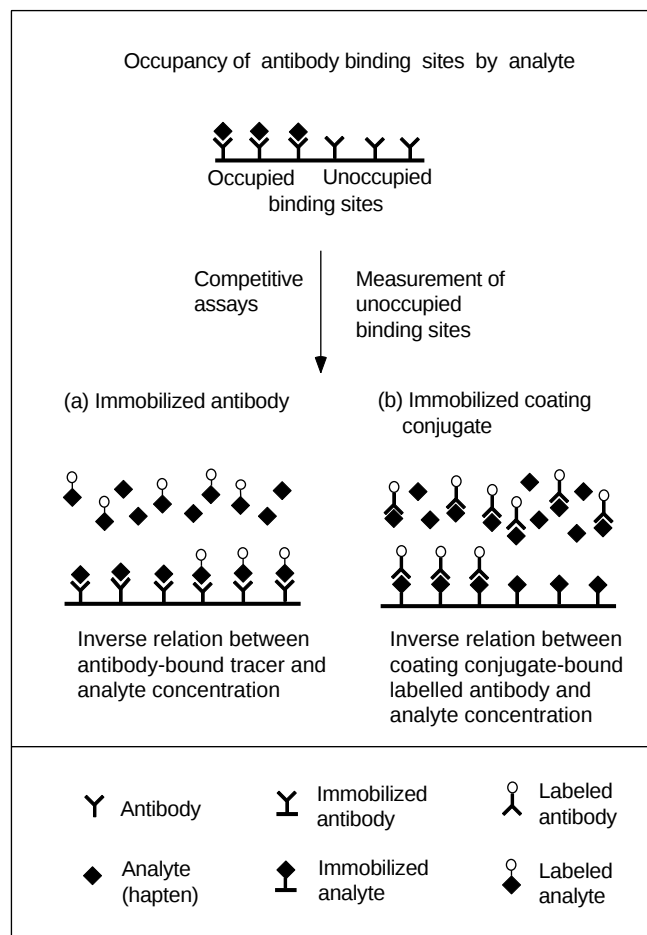


Figure 1 Principle of the competitive IA. In the first format with immobilized Ab (a) the plates are coated with Ab. Analyte and enzyme-labeled analyte compete for the Ab binding sites. In the second format a hapten–protein conjugate is immobilized in the solid phase (b). This protein conjugate and the free analyte compete for the binding sites of the Ab in solution. (Reproduced from Dankwardt and Hock⁽⁷⁾ with permission from *Food Technology and Biotechnology*.)

of the Ig class produced by the immune system against foreign material such as pathogens or xenobiotics, and bind the target substance with high selectivity and affinity. Although there are five distinct classes of Ab in most higher mammals (IgA, IgD, IgE, IgG, IgM) IgG makes up approximately 80% of the total Ig in human serum. Most IAs rely upon IgG as the major Ig.

The basic structure of an Ab molecule is shown in Figure 2. It consists of two identical heavy (H) chains and two identical light (L) chains stabilized and linked by inter- and intrachain disulfide bonds. The H- and L-chains are organized into variable and constant regions. The Ag binding site (combining site) is formed by the association of parts of the variable regions of the H- and L-chains, located at the amino terminal end. The

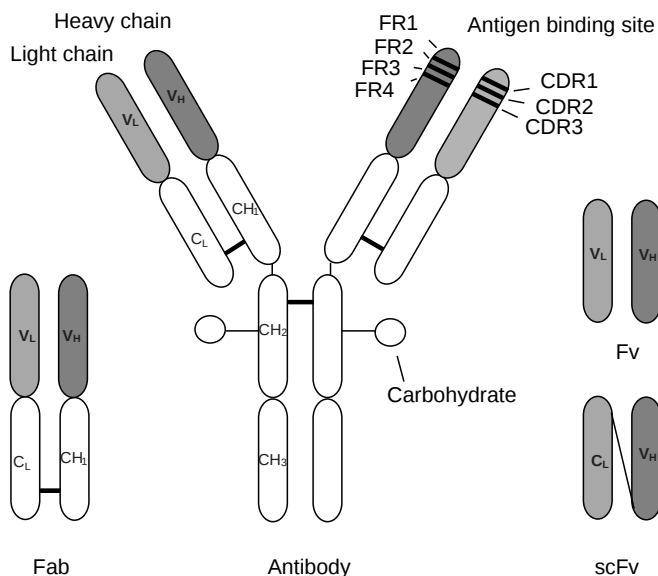


Figure 2 Structure of IgG Abs and their fragments (modified after Hock et al.⁽¹⁷⁾). scFv is the recombinant antibody fragment, a single chain fragment containing only the variable region, FR is the frame region, V_L is the variable region of light chain, V_H is the variable region of heavy chain, C_L is the constant region on the light chain, CH_1 , CH_2 , CH_3 is a constant region on the heavy chain.

variable regions of both chains are organized into three hypervariable or complementarity determining regions (CDRs) separated by four framework regions. The greatest amino acid sequence variation occurs within the CDRs whereas the framework regions are more conserved. It is assumed that the association of the CDR regions forms the combining site. The lower part of the molecule, the Fc (antibody fragment containing the crystallizable fragment) is responsible for some important biological effector functions such as complement fixation and is not necessary for Ag or hapten binding. It contains the last heavy chain domains. The whole of the Ig molecule or Ab fragments, $F(ab)_2$ and Fab (antibody fragments containing the antigen binding site(s)) can be used in IAs.

A substance that after injection into the body of a vertebrate induces a specific Ab synthesis, is called an Ag. Ags are principally macromolecules, for instance proteins, polysaccharides or nucleic acids. Synthetic polymers also belong to the antigens, i.e. they can be used as, or act as, Ags. Small molecules (haptens) such as pesticides have to be coupled to a macromolecular carrier to elicit an Ab response (see section 2.3). The ability of an Ab molecule to bind an Ag or a hapten specifically is controlled by structural and chemical interactions between the ligand and the Ab at the combining site. The Ag–Ab interaction is reversible and does not involve formation of covalent bonds.⁽¹⁶⁾ The binding is a result of a variety

of interactions such as hydrophobic, ionic, H-bonding, π - π electron interaction, and van der Waals forces. The binding energy (relative affinity of the Ab) increases with the number of specific chemical interactions between the analyte and the amino acid residues in the Ab combining site. Therefore, the selectivity and sensitivity of an IA is controlled by the nature of the Ag–Ab binding process.

2.2 Antibody Production

Ab production is conveniently carried out in warm blooded animals, e.g. rabbits, sheep, mice or chickens.⁽¹⁷⁾ Polyclonal antibodies (pAbs) are obtained from the serum and comprise a mixture of different Ab populations. Monoclonal antibodies (mAbs) consist of a single monospecific Ab population. These Abs are produced in cell culture by a single hybridoma cell derived from the fusion of B-lymphocytes with myeloma cells.⁽¹⁸⁾ The hybridoma cells can then be propagated almost indefinitely in culture and will continue to produce the Ab of the lymphocyte parent. Since an individual lymphocyte produces only a single Ab type, all of the Ab molecules produced by a hybridoma cell line derived from a single hybrid cell are identical and have the same binding properties. Therefore, the hybridoma technology guarantees the unlimited production of mAbs with constant characteristics.⁽¹⁹⁾ Owing to the great effort involved in mAb production many IAs still employ pAb. A third possibility for creating Abs has emerged, recombinant antibody (rAb) techniques. Here, Ig genes can be cloned, introduced and expressed in inexpensive and relatively simple host systems.^(20,21) Although several nonmammalian host systems (yeast, plant and insect cells) have been used to produce rAbs, the most common vehicle is *Escherichia coli*.^(22,23) The main properties of pAbs, mAbs and rAbs are listed in Table 1.

2.3 Immunogens

Most pesticides are of low molecular mass and therefore are not ordinarily antigenic. They have to be coupled to a carrier molecule, usually a protein, in order to induce an Ab response in the vertebrate immune system.⁽¹⁴⁾ The site of coupling to the carrier, the coupling procedure as well as the number of haptens bound to one carrier molecule can be of major importance for the sensitivity and the selectivity of the resulting Ab (for reviews refer to Erlanger,^(24,25) Goodrow et al.⁽²⁶⁾ and Szurdoki et al.⁽²⁷⁾).

The protein carriers used in various laboratories include globulin fractions, serum albumins of different species, hemocyanin, ovalbumin, thyroglobulin, and fibrinogen. Also nonproteinaceous carriers have been used

Table 1 Properties of polyclonal, monoclonal and recombinant Abs

Properties	pAb (Ab from serum)	mAb (Ab from hybridoma cells)	rAb (Ab produced by gene technology)
Supply	Limited and variable	Unlimited production possible	Unlimited production possible, immunization not mandatory
Uniformity	Changing properties with different sera and bleedings	Constant properties of a mAb	Constant properties of a rAb, can be changed by genetic manipulations
Affinity	Mixture of Ab with different affinities, affinity often higher with pAb	Uniformly high or low, can be selected by testing	Uniformly high or low, can be selected by testing and can be modified
Cross-reactivity	Results from different selectivities and low affinity interactions	Different, dependent upon the individual Ab	Different, dependent upon the individual Ab, can be modified
Classes and subclasses	Typical spectrum	One defined isotype	Different, depending on molecular design
Demands on Ag	High purity required for specific antisera	Impure Ags or mixture of Ags can be used for immunization, pure Ags necessary for screening	Impure Ags or mixture of Ags can be used for immunization, pure Ags necessary for screening, immunization not mandatory
Costs	Low	High	Once established, low

such as liposomes or dextran.^(28,29) Keyhole limpet hemocyanin (KLH), a protein from mollusks, is often viewed as a superior carrier because it is foreign to the vertebrate immune system.⁽³⁰⁾

Another important issue concerns the optimal number of haptens bound to the carrier protein (i.e. optimal epitope density). Highly substituted carriers usually lead to the best results. For bovine serum albumin (BSA) molar ratios of 10:1 to 20:1 (hapten:carrier) are desirable; for larger molecules such as hemocyanin, ratios of 800:1 to 1000:1 should be obtained.⁽¹⁷⁾ However, very high ratios may reduce immunogenicity because of either the changes in tertiary structure of the protein caused by masking of the essential free amino groups or the removal of critical determinant sites on the carrier by haptenic blocking.

For the production of pAbs the purity of the hapten used to prepare an immunoconjugate should be as high as possible. After synthesis of haptenic substances, closely related substances may be present in small quantities, leading to the production of nonspecific Abs and, consequently, to unwanted cross-reactivities of the antisera. This is not a problem with mAb, because a single cell line producing only one kind of Ab with the desired properties can be selected.

Another important consideration is the point of attachment on the hapten. Ab specificity is directed primarily at the part of the hapten molecule farthest away from the functional group that is linked to the protein carrier.⁽²⁵⁾ Even better specificity can be obtained with conjugates in which the hapten is coupled to the carrier via a spacer, thereby giving much better exposure of the hapten on the surface of the carrier. The spacer should be attached as far as possible from the unique

determinant structures.⁽²⁶⁾ Usually C₃–C₆ spacers are used; if the spacer is too long, it may bend back to the carrier and the hapten will not be properly exposed. Strategies for IA hapten design for the triazine, arylurea and chloroacetanilide herbicides have been summarized by Goodrow et al.⁽²⁶⁾

The functional groups of the hapten govern the selection of the method to be used to conjugate the hapten to the functional groups of the carrier. The functional groups of the protein carrier available for attachment of the haptens are the carboxyl group of the C terminal and of the aspartic and glutamic acid residues, the amino group of the N terminal and the lysine residues, the imidazol and phenolic functions of the histidine and tyrosine residues, respectively, and the sulfhydryl group of cysteine residues. General procedures for the preparation of conjugates can be found in Erlanger.^(24,25)

After coupling, characterization of the conjugates can be carried out (see Erlanger⁽²⁵⁾). Generally, the haptenic groups have an absorbance spectrum that can be differentiated from the protein carrier. Elemental analysis for the chlorine content can be carried out for some triazine conjugates. A more direct procedure is the incorporation of some radioactive hapten in the conjugation procedure. Another approach is quantitating the change in free amino groups as a result of conjugation. A recently applied technique is the determination of hapten density by matrix-assisted ultraviolet laser desorption/ionization mass spectrometry (MALDIMS)⁽³¹⁾ and electrospray ionization mass spectrometry (ESIMS).⁽³⁾ Application of energy-minimized molecular modeling methods to hapten design will help to choose the best derivatives and conjugation methods for successful Ab production.⁽³²⁾

3 IMMUNOASSAY TYPES

3.1 Assay Formats

For low-molecular-mass analytes (haptens) such as pesticides in solution, competitive tests have to be employed, using limiting Ab concentrations. The tests can be performed as homogeneous assays without separation of the reactants,⁽³³⁾ but more common are heterogeneous tests where unreacted reagents are removed before evaluation. Two different formats are available, (1) with immobilized Ab (Figure 1a) and (2) with immobilized coating conjugate (Figure 1b). In variant (1) analyte and a labeled analyte (tracer) compete for the free Ab binding sites. After removal of unbound reactants the bound tracer yields a signal that is inversely proportional to the analyte concentration. The variant (2) employs an immobilized hapten-carrier conjugate on the solid phase to which analyte and Ab are added. The Ab binds to the free analyte or to the immobilized hapten according to the concentration of the reactants. If a labeled Ab is used, the amount of Ab bound to the solid phase can be directly determined after a washing step. Alternatively, a secondary labeled Ab may be used to detect the Ab which has bound to the solid phase. The signal is inversely proportional to the amount of free analyte in the sample. Very sensitive competitive IAs have been developed with DLs between 1 and 50 ng L⁻¹, for example for the triazines and urea herbicides.^(34–36)

An example for a homogeneous assay system is the polarization fluoroimmunoassay (PFIA). PFIA measures the increased polarization of fluorescence when a fluorophore-labeled hapten (tracer) is bound by a specific Ab, and the decreased signal when free analyte competes

with the tracer for binding.⁽³⁷⁾ While these assays are easy to carry out and very suitable for automation, they usually show a lower sensitivity than EIAs, e.g. for simazine a DL of 5 µg L⁻¹ was observed.⁽³⁷⁾

Noncompetitive assays can only be applied for high-molecular-mass analytes with more than one antigenic determinant (i.e. Ag) or low-molecular-mass analytes (haptens) bound to a solid phase, exposing the antigenic determinant. They work with an Ab excess. Noncompetitive IAs have been employed for the detection of soil-bound pesticides.^(38,39) In this case the soil particles, to which the pesticide residues have bound, form the solid phase, and the residues can be detected by a labeled Ab specific to the analyte.

3.2 Labels

Depending on the label, IAs are classified in different groups. Radioisotopes are used in RIAs, enzymes in enzyme-linked immunosorbent assays (ELISAs) or EIAs, fluorophores in FIAs or PFIA and chemiluminescent compounds in CLIAs. Additional types of IA exist, but are not very common in pesticide analysis. A more detailed description of these IAs can be found in Gosling.⁽⁴⁰⁾

EIAs are most commonly used in pesticide analysis as they avoid the necessity of working with radioactive material and low DLs can be reached. Simple and cheap photometers which give an extremely rapid measurement capability and long-lasting stability of the colored product after the reaction has stopped make EIA superior to fluorimetry or luminometry, even though with these methods lower DLs may be reached.⁽³³⁾ Enzymes

Table 2 Enzyme systems commonly used for EIAs

Enzyme	Source	Molecular weight	pH optimum	Colorimetric substrates	Fluorometric substrates	Luminometric substrates
Alkaline phosphatase	Calf intestine	100 000	9–10	<i>p</i> -Nitrophenyl-phosphate	4-Methylumbelliferyl-phosphate	Adamantyl-1,2-dioxyethane Phenylphosphate-substituted dioxyethane
β-Galactosidase	<i>Escherichia coli</i>	540 000	6–8	<i>o</i> -Nitrophenyl-β-D-galactopyranoside Chlorophenolic red-β-D-galactopyranoside	4-Methylumbelliferyl-β-D-galactopyranoside	–
Peroxidase	Horseradish	40 000	5–7	2,2'-Azino-di(3-ethylbenzthiazoline sulfonic acid-6) (ABTS)/H ₂ O ₂ 3,3'-5,5'-Tetramethylbenzidine (TMB)/H ₂ O ₂ <i>o</i> -Phenylendiamine (OPD)/H ₂ O ₂	<i>p</i> -Hydroxyphenyl-acetic acid <i>p</i> -Hydroxyphenyl-propionic acid	Luminol

Table 3 Solid phases used for EIAs

Material	Form	Binding	Capacity
Polystyrene	Microtiter plates, tubes, pins, beads	Noncovalent	250–500 ng cm ²
Polyethylene	Tubes	Noncovalent	ca. 300 ng cm ⁻²
Polypropylene	Microtiter plates	Noncovalent	ca. 300 ng cm ⁻²
Polyvinylchloride and similar	Microtiter plates, membranes	Noncovalent	ca. 300 ng cm ⁻² (plates)
Polycarbonate	Beads, membranes	Noncovalent	ca. 300 ng cm ⁻² (beads)
Nitrocellulose	Microtiter plates, membranes	Noncovalent	ca. 100 µg cm ⁻²
Protein A coated	Microtiter plates, beads	Noncovalent	20 mg mL ⁻¹ (for Ab only)
Activated polymer, with amino or carboxyl groups	Microtiter plates, beads	Covalent, using bifunctional reagents	2×10^{13} – 1×10^{14} reactive sites/cm ²
Magnetic	Beads	Depends on the surface of the beads	Varies

commonly used as labels in heterogeneous EIA are listed in Table 2.

The following requirements are necessary for the use of an enzyme as a marker:

- (1) high specific activity (turnover number) of free enzyme and after labeling,
- (2) availability of soluble, purified enzyme at low cost and reproducible quality,
- (3) high stability in free and conjugated form under storage and assay conditions,
- (4) presence of reactive groups for covalent linkage to hapten,
- (5) simple and gentle conjugation methods,
- (6) inexpensive and stable nontoxic substrates with formation of stable chromogenic, fluorogenic and/or chemiluminogenic products.

3.3 Solid Phases

IAs are mainly carried out in 96-well polystyrene, polyethylene, polypropylene or polyvinyl microtiter plates, owing to the easy separation of the reactants in a washing step, but polystyrene tubes, beads or pins are also available (Table 3). The plastic plates are of comparatively low binding capacity and low surface area to volume ratio. High-binding supports include agarose and cellulose. Particulate solid phases are very efficient, because they become scattered throughout the reaction mixture and have a much higher surface area to volume ratio.⁽⁴¹⁾ For example, many chemically different beads are available (e.g. polystyrene, latex, polycarbonate and copolymer beads). Immunological reagents are bound to the beads in a similar manner as they are to microtiter plates. Separation of bound and free reagents occurs by washing and centrifuging. IAs using magnetic beads employ a magnet for the separation step.⁽⁴²⁾ Abs and Ags may be immobilized to some solid phases via

covalent binding. Those solid supports contain amino or carboxy groups on a modified surface through which the immunoreagents can be bound by water-soluble carbodiimides or bifunctional reagents such as glutaraldehyde.

Other solid-phase supports for IAs are membranes. They can be used for dip sticks, which are incubated for a short time in the solution⁽³⁶⁾ or for dot blots and immunofiltration tests. Here the reactants are filtered through the membrane.^(43,44) The test principle is the same as for the microtiter plate tests but the reaction time is much shorter owing to the high surface area of the membrane and the short distance between reaction partners. Application of remission measurements yields a proportional relationship between analyte and remitted light. By using a pocket reflectometer, this set-up is ideally suited for field-monitoring purposes.^(45,46)

4 PROPERTIES OF COMPETITIVE IMMUNOASSAYS

4.1 Dose–Response Curve

In IAs the signal produced is inversely correlated to the analyte concentration in the sample (Figure 3a). The typical dose–response curve is of sigmoidal shape when the signal is plotted versus the logarithm of the analyte concentration. A linear range is obtained around the middle of the test (IC₅₀, middle of assay, concentration of analyte that causes 50% inhibition), which should be used for determinations. Within this working range, the change in absorbance is linearly correlated to the analyte concentration. The linear part of the curve is confined by the upper and lower limits of quantification. These are the cut-off values above or below which quantitative results can be obtained with a stated relative precision, or specified degree of confidence in real samples.⁽⁴⁷⁾ The experimental errors

increase toward these limits. Consequently, the most precise measurements are obtained in the region close to the middle of the test.

The DL (or least detectable dose) is the smallest concentration of the analyte that produces a signal which can be significantly distinguished from zero for a given sample matrix with a stated degree of confidence. Very often a dose is selected which inhibits 10–20% of enzyme tracer from binding with the Ab or the dose calculated after subtraction of two or three times the standard deviation from the mean measurements of the zero dose signal.⁽³⁾

Linearization of the calibration curve is useful for many purposes, for instance, for the direct comparison of curves if matrix effects are evaluated. Absorbance curves can be normalized by converting the absorptions to $%B/B_0$ values. These can be expressed as the ratio of bound tracer in the presence of hapten to bound tracer in the absence of hapten and lies between 100% ($=A_0$, the upper asymptote of the curve) and 0% ($=A_{\text{Excess}}$, the lower asymptote) (Figure 3b). They are calculated by Equation (3):

$$\frac{\%B}{B_0} = \frac{A - A_{\text{Excess}}}{A_0 - A_{\text{Excess}}} \times 100 \quad (3)$$

Linearization can be obtained by various mathematical transformations.⁽⁴⁷⁾ Usually, IAs are evaluated with commercial IA programs, often based on logistic models (cf. Rodgers⁽⁴⁸⁾ and Dudley et al.⁽⁴⁹⁾), e.g. four-parameter models or the more simple logit-log transformation (two-parameter model, Figure 3c) which can also be carried out with a calculator (s), Equation (4):

$$\text{logit } \frac{\%B}{B_0} = \ln \frac{\%B/B_0}{100 - \%B/B_0} \quad (4)$$

4.2 Quality Control

Precision and accuracy of IA are important properties which deserve special attention. The quality and stability of the employed material (microtiter plates, pipettes) and reagents (e.g. Abs, enzyme tracer or buffers), play a crucial role.⁽⁵⁰⁾ The long-term stability of reagents has to be ensured, e.g. by freeze-drying of Abs and, if necessary, addition of stabilizing components to the test reagents such as the enzyme tracer.⁽⁵¹⁾

In spite of the simple handling of the assays, expert knowledge is required, especially to recognize and remove incident errors. Therefore, IAs should be performed by trained personnel. The development of simple and rapid assays, such as dip-stick assays or immunofiltration tests reduces the requirement for trained users, but one has still to be aware of potential problems such as interferences from the sample matrix.

The precision of an IA is defined as the extent to which replicate analyses of a sample agree with each other.

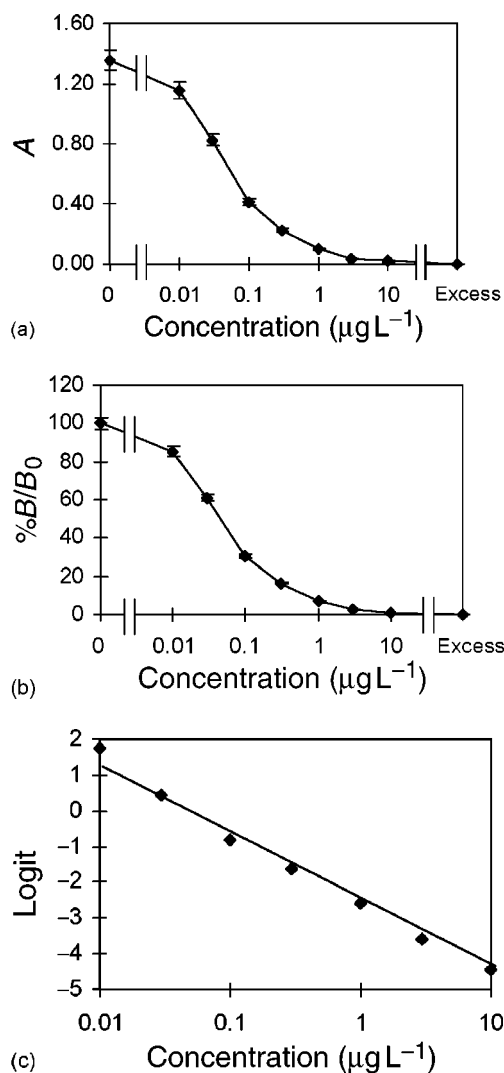


Figure 3 EIA for the determination of atrazine using pAb. (a) Absorption curve (means of three determinations $\pm$ standard deviations), (b) B/B_0 curve, and (c) logit/log transformation by the two-parameter fit.

The reproducibility is the ability to yield the same results within analyses, between analyses, and between operators. The investigation of the variability of an IA gives valuable information about the consistency of the test. Coefficients of variation (CV) of IA measurements are usually between 10 and 20% for an optimized assay,^(52,53) although more precise results can be obtained.^(54,55) Same-day and day-to-day CV of samples have been determined in different matrices.^(53,56) Interlaboratory tests of the same IA as that carried out by Hock and the IA Study Group⁽⁵⁷⁾ and Hayes et al.⁽⁵⁸⁾ for the investigation of triazines help to evaluate the general applicability of a test. However, several conditions like exact description of the assay including calibration curves, DLs, cross-reactivities, a working range close to the middle of the

test, enough parallel measurements, etc. must be met (see also AOAC (Association of Official Analytical Chemists) criteria). Meanwhile, standardized procedures for IAs in water are adopted by AOAC International and have been established in Germany as a prenorm.^(50,58)

A validation of the results obtained by IA should be carried out. To a limited extent this can be done by IA itself. Dilution of the samples as well as spiking of the authentic sample with known amounts of the contaminant can be used to check whether the matrix interferes with the IA.⁽⁵⁹⁾ However, spiked samples do not completely mimic real unknown samples. They do not contain potential metabolites of the contaminant nor residues from other compounds which may be present in real samples. Furthermore, spiked samples cannot be a model for aged residues which are more difficult to extract and detect because, for example, they may have bound to soil constituents. Therefore, an IA should also be validated by a different established method like high-performance liquid chromatography (HPLC), gas chromatography (GC) or GC/MS (gas chromatography/mass spectrometry). Many groups have used this approach and have usually obtained correlation coefficients of >0.9 .^(60–63) Often a slight overestimation of the IA in comparison with HPLC or GC is observed owing to cross-reactivities of the Ab or matrix effects.

4.3 Cross-reactivity

Depending on the conjugate used for immunization and the class of chemicals under investigation, cross-reactivities of the Ab with haptens similar to the analyte are frequently observed (see e.g. Hock⁽¹⁴⁾ and Harrison et al.⁽⁶⁴⁾). Therefore, it should be checked which compounds cross-react to what degree with the Ab. This is usually done by comparing the standard curves of the analyte under investigation with similar haptens, using analyte concentrations at 50% of the inhibition curve as the reference. However, cross-reactivity with a certain analyte is not the same over the whole range of a standard curve. Often higher cross-reactivities can be observed at low concentrations of the cross-reacting analyte.⁽³⁾ Therefore, it has been recommended that cross-reactivities be measured at different concentrations over the range where the assay is suitable.⁽⁶⁵⁾

If an Ab is selective for a single compound, it is regarded as monospecific⁽⁶⁶⁾ (Figure 4). An Ab that recognizes several compounds to the same extent (e.g. a group of s-triazines), can be used for the screening of a class of herbicides⁽⁶⁷⁾ (group-specific Ab, Figure 4). If cross-reacting compounds are not expected in the samples, because the compounds are not licensed (e.g. propazine in most European countries), a group-specific Ab can also be used for quantitative measurements of one compound.⁽⁶⁸⁾

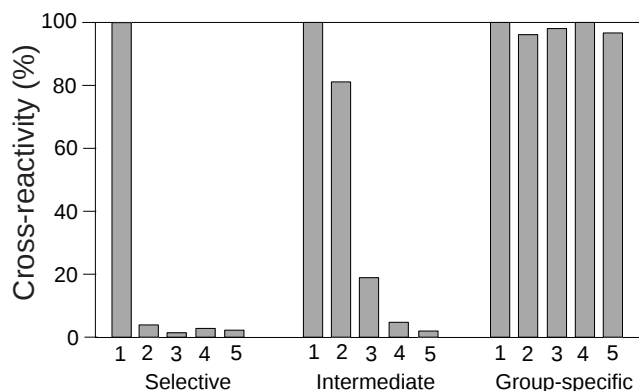


Figure 4 Selective, intermediate and group specific Abs. This example uses Abs which have been produced against hapten 1. Substances 2–5 are assumed to be cross-reacting haptens (modified after Hock et al.⁽¹⁴⁾).

Strong cross-reactivities of an Ab to unexpected metabolites, for example, can produce false positive values. An Ab for alachlor was found to react very strongly to the sulfonic acid metabolite using an alachlor screening kit.⁽⁶⁹⁾ This problem could be solved, however, by using solid-phase extraction (SPE) prior to IA and sequential elution of the two compounds with different organic solvents.

4.4 Sample Preparation and Matrix Effects

Samples can contain compounds in addition to the target analyte, which may interfere with the test. Several groups investigated the influence of ions on EIAs.^(70–72) Ruppert et al.⁽⁷⁰⁾ observed an inhibition by several anions like azide, which inhibits the peroxidase by binding to the heme group of the enzyme. Most cations did not have an effect except for Ca^{2+} , which leads to an activation of the peroxidase. No interference by different ions such as nitrate, copper, magnesium etc. up to a concentration of 250 ppm was detected in an EIA for pentachlorophenol in water.⁽⁷¹⁾ While ions may inhibit the enzyme used as a label or lead to precipitates by reacting with the buffer components, humic substances present in water or soil extracts may bind nonspecifically to the Ab and thereby interfere with the specific binding of the analyte.⁽⁷³⁾ These reactions may lead to false positive results. Water samples from forest stands or soil extracts particularly contain a high content of organic compounds such as humic acids (HAs).

Matrix effects in food samples frequently occur owing to colored extracts or to the content of lipids, proteins or polyphenols that may be coextracted during sample preparation.⁽⁷⁴⁾ As food samples usually have to be extracted prior to immunochemical analysis, the method of analyte extraction is of great importance. Analytes

that are water soluble and can be efficiently extracted in aqueous buffer will have the most direct extraction method and eliminate the need for organic solvents. However, many pesticides are not readily water soluble and must be extracted with an organic solvent.⁽⁵⁾ For the extraction of pesticides from solid foods a variety of solvents have been tested, such as acetone, ether, petroleum ether, methanol, acetonitrile or hexane.⁽⁷⁵⁾ Direct analysis of extracts by IA requires the use of solvents that are miscible with water and (at low concentrations) are nondenaturing to proteins such as Ab. IAs are to a certain degree tolerant to a variety of solvents, but each system must be tested to determine which solvent can be accepted and to what extent (for example Hill et al.,⁽⁷⁵⁾ Nugent⁽⁷⁶⁾ and Schneider and Hammock⁽⁷⁷⁾). Usually the extracts are further diluted with water prior to the EIA, but an EIA for parathion was developed, in which the analyte dissolved in hexane could be directly measured in the EIA without prior removal of the hexane. This was achieved by using Ab encapsulated in reverse micelles composed of Aerosol T with aqueous centers.⁽⁷⁸⁾ However, a 10^4 -fold decrease in sensitivity was observed.

In some cases a cleanup step is introduced, in which the analyte of interest is separated from the matrix. This can be carried out by C_{18} -columns or immunoaffinity columns.^(69,79,80) A very interesting approach is the application of supercritical fluid extraction (SFE) prior to immunoanalysis. These methods generally employ CO_2 or CO_2 containing various modifiers.^(5,81)

Some problems with interfering ions can be solved by changing the buffer of the assay system so that no precipitates may be formed.⁽⁷⁰⁾ Addition of BSA to the plates prior to the addition of the standard and sample solutions⁽⁸²⁾ or to the enzyme tracer⁽⁷³⁾ greatly reduces the influence of humic and fulvic acids on the EIA. It may also be helpful to switch to a different batch of Abs or a different assay kit, as different Abs may show different sensitivities to interfering substances. The buffering capacity of the assay buffer should also be checked, as some water or food samples may show relatively low pH values. No effects, however, were observed between pH 3 and 10 by different investigators.^(56,67,83)

5 APPLICATION TO ENVIRONMENTAL SAMPLES

IAs have been developed for many environmental contaminants during the 1990s. A list of several IAs described in the literature can be found in Table 4. Most of them have been developed in laboratories, showing the increasing importance of immunochemical

methods in residue analysis. Not all of them are commercially available. Available commercial IAs have been listed in e.g. Dankwardt et al.,⁽²⁾ Hennion and Barcelo,⁽³⁾ Knopp,⁽⁸⁾ but a lot of movement has been observed in environmental IA markets, leading to the disappearance of IA companies. At the moment IAs for environmental contaminants can be obtained for example from Strategic Diagnostics Inc. (Newark, DE, USA, sells former Ensys, Millipore and Ohmicron kits) and EnviroLogix (Westbrook, MA, USA).

5.1 Water

EIA have been used intensively for the determination of pesticides in surface and rainwater^(56,60,69,211–217) and groundwater.^(60,69,214,218,219) A substantial number of these studies were carried out for triazine herbicides.^(56,60,211,212,214,216,218,219) This illustrates the widespread occurrence of these herbicides in the aquatic environment. Many groups have used commercial test kits, which allow the investigation of samples without time-consuming Ab production. Thurman et al.,⁽⁶⁰⁾ for example, used a Res-I-Mune kit (ImmunoSystems) for the investigation of triazines in surface and groundwater. The EIA was compared to GC/MS results obtained from samples that were extracted by SPE. Correlation coefficients between 0.91 and 0.95 were obtained after introducing cross-reactivity factors for each of the triazines in order to calculate a sum parameter for the GC. The majority of the samples contained only atrazine (up to $3\mu\text{g L}^{-1}$). Therefore, the EIA results corresponded well with the atrazine concentrations obtained by GC/MS.

Mouvet et al.⁽²²⁰⁾ compared four commercially available test kits and one in-house developed assay for the determination of triazines in surface and groundwater. Operational characteristics, cross-reactivity, sensitivity, CV and agreement with GC/LC (gas chromatography/liquid chromatography) measurements were investigated. DLs were determined between 0.003 and $0.07\mu\text{g L}^{-1}$. Intra-assay CVs were below 7% for all tests, interassay CVs below 20%. Correlation studies between the EIA kits and GC/LC were carried out for samples from different water matrices. Depending on the water source, different levels of significance were observed with different tests. The best results were obtained for surface water, while not all kits showed a good agreement for lysimeter samples.

Apart from the triazines some other pesticides were investigated in water samples, also using commercial test kits. Alachlor was determined in ground and surface water using commercial tests.⁽²¹³⁾ SPE was carried out prior to EIA to remove interfering substances and to concentrate the analyte. Concentrations of up to $0.8\mu\text{g L}^{-1}$ were observed, and a comparison with GC/MS showed a

Table 4 Pesticide IAs described in the literature

Pesticides	Test format	Ab	Range, DL, or middle of test (IC ₅₀)	Ref.
Herbicides				
Alachlor	EIA	p	0.2–8 µg L ⁻¹	84
	EIA	p	0.1–10 µg L ⁻¹	85
	EIA	p, m	0.2–8 µg L ⁻¹	86
Amitrole	EIA	p	1.7–4200 µg L ⁻¹	87
Atrazine	CLIA	p	25–500 ng L ⁻¹	88
	EIA	p	0.5–10 µg L ⁻¹	89
	EIA	p	0.01 µg L ⁻¹ (DL)	90
	EIA	m	0.03–1 µg L ⁻¹	35
	EIA	p	0.2–100 µg L ⁻¹	64
	EIA	p	0.011–33 µg L ⁻¹	91
	EIA	m	0.05–3 µg L ⁻¹	92
	EIA	p, m	0.1–100 µg L ⁻¹	93
	EIA	p	0.5–10 µg L ⁻¹	94
	EIA	m	0.05 µg L ⁻¹ (DL)	95
	EIA	m	0.01–10 µg L ⁻¹	77
	EIA	p	0.03–3 µg L ⁻¹	96
	EIA	p	1–1000 ng L ⁻¹	34
	EIA	p	2–24 µg L ⁻¹	97
Bentazon	EIA	p	0.1–160 µg L ⁻¹	98
Bromacil	EIA	p	0.01–1 µg L ⁻¹	99
	EIA	p	160–480 µg L ⁻¹	100
Chlorodiamino-s-triazine	EIA	p	0.1 µg L ⁻¹ (DL)	101
Chlorsulfuron	EIA	p	2–250 µg L ⁻¹	102
Clomazone	EIA	p	0.5–500 µg L ⁻¹	103
	EIA	p	0.035–3 µg L ⁻¹	104
Cyanazine	EIA	p	0.5 µg L ⁻¹ (DL)	105
	EIA	p	0.5 µg L ⁻¹ (DL)	106
	EIA	p	0.01–100 µg L ⁻¹	107
Diethylatrazine	EIA	p	10–75 µg L ⁻¹	108
Diclofop-methyl 2,4-D	EIA	p	50–5000 µg L ⁻¹	109
	EIA	m	2–20 µg L ⁻¹	110
	RIA	p	0.1–10 mg L ⁻¹	111
	EIA	p	0.05–10 mg L ⁻¹	111
	RIA	p	5–250 µg L ⁻¹	112
	PFIA	m	0.6 µg L ⁻¹ (DL)	113
	RIA	p	1–1000 µg L ⁻¹	114
	EIA	m	0.096 µg L ⁻¹ (DL)	115
	PFIA	p	0.01–100 µg mL ⁻¹	116
	EIA	m	2 µg L ⁻¹ (IC ₅₀)	117
Diuron	EIA	p	0.05–1 µg L ⁻¹	118
	EIA	p	0.22–17.6 µg L ⁻¹	119
Hexazinone	EIA	m	0.03–1 µg L ⁻¹	120
	EIA	m	0.05 µg L ⁻¹ (DL)	95
	EIA	p	0.01–10 µg L ⁻¹	66
	EIA	p	3–300 µg L ⁻¹	121
Imazamethabenz	EIA	p	0.5–32 µg L ⁻¹	122
Imazaquin	EIA	p	0.45–25 µg L ⁻¹	123
Isoproturon	EIA	p	0.01–10 µg L ⁻¹	124
	EIA	m	20–250 µg L ⁻¹	125
	EIA	NA	0.02–1 µg L ⁻¹	126
Maleic hydrazide	EIA	m	0.01–11 µg mL ⁻¹	127
MCPB	EIA	p	0.03–0.9 µg L ⁻¹	128
Metazachlor	EIA	p	10–1000 ng L ⁻¹	129

(continued overleaf)

Table 4 (continued)

Pesticides	Test format	Ab	Range, DL, or middle of test (IC ₅₀)	Ref.
Methabenzthiazuron	EIA	p	0.05–10 µg L ⁻¹	130
Metolachlor	EIA	m	0.05–10 µg L ⁻¹	131
	EIA	p	0.05–5 µg L ⁻¹	132
	EIA	p	6 µg L ⁻¹ (IC ₅₀)	133
Molinate	EIA	p	3–2000 µg L ⁻¹	134
	EIA	p	10–500 µg L ⁻¹	135
Monuron (Diuron)	EIA	p	0.08–5 µg L ⁻¹	136
Monuron (Diuron, Linuron)	EIA	p	0.05–5 µg L ⁻¹	36
Norflurazon	EIA	p	1–1000 µg L ⁻¹	137
Paraquat	RIA	m	0.46–165 µg L ⁻¹	138
	FIA	p	20–2000 µg L ⁻¹	139
	RIA	p	1–100 µg L ⁻¹	140
	EIA	p	20–200 ng L ⁻¹	141
	RIA	p	50–1600 µg L ⁻¹	142
	EIA	m	10–100 µg L ⁻¹	143
	EIA	m	0.8–12 µg L ⁻¹	144
	EIA	p	20 ng L ⁻¹ (DL)	145
	EIA	p	1–100 µg L ⁻¹	146
	EIA	p	0.1–27 µg L ⁻¹	147
Picloram	EIA	m	1–200 µg L ⁻¹	148
	EIA	p	5–5000 µg L ⁻¹	148
	RIA	p	0.05–5 mg L ⁻¹	149
Propazine	EIA	m	0.02–3 µg L ⁻¹	120
Simazine	EIA	p	0.1–10 µg L ⁻¹	150
	PFIA	p	3–1000 µg L ⁻¹	150
	EIA	p	0.05–10 µg L ⁻¹	151
Terbuthylazine	EIA	m	0.14–10 µg L ⁻¹	152
Terbutryn	EIA	m	0.05–1 µg L ⁻¹	153
	EIA	p	0.1–600 µg L ⁻¹	154
	EIA	m	0.1–10 µg L ⁻¹	95
Thiobencarb	EIA	p	20–1000 µg L ⁻¹	155
Triasulfuron	EIA	p	0.004–40 µg L ⁻¹	156
	EIA	m	0.01–1 µg L ⁻¹	157
2,4,5-T	RIA	p	1–1000 µg L ⁻¹	158
Trifluralin	EIA	p	0.1–1 mg L ⁻¹	159
Insecticides				
Aldicarb	EIA	p	0.3–40 µg L ⁻¹	160
	EIA	p	1–100 µg L ⁻¹	161
Aldrin	RIA	p	0.7–35 ng	162
Azinphos-methyl	EIA	m	0.4–20 µg L ⁻¹	163
S-Bioallethrin	EIA	m	1–250 µg L ⁻¹	164
	EIA	p	0.8–28 µg L ⁻¹	165
	RIA	p	0.03–3 µg	166
	EIA	p	0.01–100 mg L ⁻¹	167
Bioresmethrin	EIA	p	50–10 000 µg L ⁻¹	168
Carbaryl	EIA	m	0.02–20 µg L ⁻¹	169
	EIA	p	0.05–10 µg L ⁻¹	170
Carbofuran	EIA	p	0.056–5 µg L ⁻¹	72
Chlorpyrifos	EIA	m	2–45 µg L ⁻¹	55
DDA	EIA	p	10–100 µg L ⁻¹	171
DDT	EIA	m	2–11 nM L ⁻¹ (IC ₅₀)	172
Dieldrin	RIA	p	0.08–38 ng	162
Diflubenzuron	EIA	p	0.5–15 µg L ⁻¹	173
Endosulfan	EIA	p	3–400 µg L ⁻¹	174
	EIA	p	0.2–10 µg L ⁻¹	175
	EIA	p	3–500 µg L ⁻¹	176

Table 4 (continued)

Pesticides	Test format	Ab	Range, DL, or middle of test (IC ₅₀)	Ref.
Fenitrothion	EIA	p, m	1–1000 µg L ⁻¹	177
Chlorpyrifos	EIA	p	0.05–2.5 µg L ⁻¹	178
Heptachlor	EIA	m	10–200 µg L ⁻¹	179
Methoprene	EIA	p	0.1–100 µg L ⁻¹	75
	EIA	p	1–10 µg L ⁻¹	180
1-Naphthol	EIA	p	10–1000 µg L ⁻¹	181
Parathion	RIA	p	100 µg L ⁻¹ (DL)	182
	EIA	p	0.1 µg L ⁻¹ (DL)	183
	EIA	p	0.1–10 µg L ⁻¹	78
	EIA	p	0.2 µg L ⁻¹ (DL)	184
Paraoxon	EIA	m	10–100 µg mL ⁻¹	185
	RIA	p	0.2–19 ng L ⁻¹	186
	EIA	p	28–275 µg L ⁻¹	186
	EIA	p	0.03 µg L ⁻¹ –275 mg L ⁻¹	187
PCP	EIA	p	0.3–150 µg L ⁻¹	188
	EIA	p	0.1–10 µg L ⁻¹	71
	EIA	p	25 µg L ⁻¹ (DL)	189
Permethrin	EIA	p	10–6000 µg L ⁻¹	190
	EIA	m	15–100 µg L ⁻¹	191
	EIA	m	1.5–90 µg L ⁻¹	192
Pyrimiphos-methyl	EIA	p	30 µg L ⁻¹ (DL)	193
Quassin	EIA	p	0.05–3 µg L ⁻¹	194
3,5,6-Trichloro-2-pyridinol	EIA	m	0.25 µg L ⁻¹	195
Fungicides				
Benomyl	EIA	m	0.1–1 µg L ⁻¹	196
	PFIA	p	0.1–10 µg L ⁻¹	197
	RIA	p	1.25 µg L ⁻¹ (DL)	198
	EIA	p	0.38 µg L ⁻¹ (DL)	199
	EIA	p	0.35 µg L ⁻¹ (DL)	200
Benzimidazole	EIA	m	1–20 µg L ⁻¹	201
Captan	EIA	p	1–200 µg L ⁻¹	202
Chlorothalonil	EIA	p	0.07 µg L ⁻¹ (DL)	203
Fenpropimorph	EIA	p	0.1–0.8 µg L ⁻¹	204
Iprodione	EIA	p	0.1–10 mg kg ⁻¹	205
Metalaxyl	EIA	p	0.06–1 µg L ⁻¹	206
Myclobutanil	EIA	p	0.5–50 mg L ⁻¹	207
Procymidone	EIA	p	1 µg L ⁻¹ (DL)	141
Thiabendazole	EIA	m	0.5–10 µg L ⁻¹	208
	EIA	p	0.03–0.5 µg L ⁻¹	141
Triadimefon	EIA	p	2 µg L ⁻¹ (DL)	209
Triazole	EIA	p	10–1200 µg L ⁻¹	210

MCPB, 4-(4-chloro-2-methylphenoxy)butyric acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; DDA, bis(*p*-chlorophenyl)acetic acid; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; 2,4-D, 2,4-dichlorophenoxyacetic acid; PCP, pentachlorophenol. NA, not available.

correlation coefficient of 0.95 with a slight underestimation by EIA. The occurrence of carbaryl was determined by Marco et al.⁽²²¹⁾ in wellwater from Spain with their own assay and compared with a commercial test kit. Both IAs yielded a good agreement with conventional methods. Concentrations of 0.08–1.37 µg L⁻¹ were observed. Two commercial test kits were used in the Netherlands to determine 2,4-D concentrations in the rivers Rhine and

Meuse.⁽²¹⁷⁾ By diluting the kit standards with kit zero-buffer and calculating the DL on the basis of the error in the zero-standard, the DL as originally indicated by the manufacturer was significantly lowered. The water matrix substantially affected the recovery of 2,4-D with one assay kit, yielding unexpectedly low recoveries in demineralized and tap water. However, similar results were obtained by EIA and GC/MS for spiked samples from the river Rhine

(with a slope of about 1 and $r = 0.99$). Routine samples were also analyzed and yielded analyte concentrations mostly below the DL ($0.03\text{--}0.05\ \mu\text{g L}^{-1}$).

5.2 Soil

Many studies for pesticides in soil have been carried out for triazines.^(38,73,222–227) Other analytes include carbofuran,⁽⁷²⁾ chlorpyrifos,⁽¹⁷⁸⁾ hexazinone,⁽¹¹⁹⁾ metolachlor,⁽²²⁷⁾ 4-nitrophenol,⁽¹⁸⁴⁾ PCP,⁽⁷¹⁾ and triasulfuron.^(157,228)

Usually, the pesticides are extracted from soil with organic solvents such as acetone, ethyl acetate or methanol. In some cases, for example in the investigation of the quite water-soluble atrazine, extraction with water was sufficient. In one study no difference was observed in EIA measurements after extraction of atrazine from corn fields with water or acetone.⁽²²⁵⁾ Owing to the extraction with water it was very easy to apply the samples directly to the IA. However, in most cases the sensitivity of the assays is high enough to dilute organic solvent extracts sufficiently so that they do not interfere with the assay. The use of supercritical fluids for soil extraction is becoming increasingly popular. The low critical temperature (e.g. $31\ ^\circ\text{C}$ for CO_2) means that low extraction temperatures can be used to recover thermally unstable solutes.^(184,229) Wong et al.⁽¹⁸⁴⁾ compared SFE with solvent extraction using ethyl acetate for the investigation of parathion and 4-nitrophenol from soil. The extracts could be measured directly after SFE by IA or GC without solvent exchange. Similar concentrations were observed by both extraction methods.

The use of IA with magnetic particles has been described for the investigation of PCP, carbofuran, and triasulfuron in soil.^(71,72,228) The dispersion of the covalently immobilized Ab on the surface of the magnetic beads results in a more even distribution in the sample and leads to a lower variability of measurements, which is usually higher in soil extracts than in water samples.

Immunochemical methods have also been applied to the detection of bound pesticide residues in soil. These are formed by binding of pesticides to the organic matter of the soil, mainly humic and fulvic acids, and cannot be analyzed using common extraction and assay methods. Hahn et al.⁽²²³⁾ used Fab fragments labeled with a fluorescent dye to detect nonextractable residues of atrazine in soil from corn fields. The fluorescence signal obtained was related to the amount of bound atrazine in native soil samples determined by GC after supercritical methanol extraction. A noncompetitive sandwich IA for the analysis of bound residues based on HA–Ab and triazine Ab was developed by Ulrich et al.⁽³⁸⁾ HA was extracted from soil, bound to the plates by the HA–Ab and the nonextractable triazine residues were detected by

a peroxidase-labeled Ab against triazines. Concentrations of bound atrazine were found in the region of 2 mg per kg soil in corn fields with a long history of atrazine application. A competitive EIA was used by Dankwardt et al.⁽²²⁵⁾ for the investigation of bound residues. Free atrazine residues were removed from organic matter extracts by SPE and the extracts were then measured by EIA.

5.3 Food

A variety of pesticides has been determined in food samples by EIA. In many cases Abs against the target compound were developed in-house. Mainly pAbs were still used, but mAbs were applied for the determination of atrazine, benzimidazoles, thiabendazoles and carbaryl.^(46,201,208,230)

Peel of apples, potatoes, oranges, grapefruits and bananas were investigated for thiabendazole residues employing an EIA with mAb.⁽²³¹⁾ Residues were extracted by soaking peel overnight in 80% methanol and filtering the decanted supernatants. Most of the thiabendazole was extracted within 1 h, but an extraction time of 16 h was chosen as uniformly high recoveries from all matrices were obtained. A 20-fold dilution eliminated significant matrix effects. The EIA had a DL of 0.1 ppm in peel samples, corresponding to 10–40 ppb in the whole fruit or tuber. Results obtained by EIA were compared with HPLC analyses. Although the EIA values were higher compared with the HPLC results, the two sets of data correlated well. The higher values by EIA were attributed to the loss of thiabendazole during sample work-up for HPLC and the high dilutions necessary for EIA.

Triadimefon was added to different food commodities such as apples, pears, pineapples and grapes. Fungicide residues were determined by EIA and GC after extraction with ethyl acetate or methanol.⁽²⁰⁹⁾ Ethyl acetate was required to extract grapes for the EIA procedure, since methanol resulted in coextractives which gave too low values. Methanol, however, yielded good recoveries at 0.5 ppm and above for other commodities and is preferable to ethyl acetate because it avoids an evaporation step. Low recoveries were obtained at 0.1 ppm and were not improved using ethyl acetate. On the whole, recoveries obtained by EIA correlate well with those measured by GC. The middle of the test was observed at $2.4\ \mu\text{g L}^{-1}$.

A mAb-based EIA was applied to the determination of carbaryl in apple and grape juices.⁽²³⁰⁾ The juices were used without any sample pretreatment and spiked with different carbaryl concentrations. The influence of matrix dilution was investigated using different dilutions of the samples. For proper analysis the samples should be diluted at least 1:5–1:10. With a dilution of 1:100

the most accurate and precise results were obtained. Therefore $2\text{--}5\text{ }\mu\text{g L}^{-1}$ are considered the lowest cabaryl concentrations in juices that can be reliably measured with the EIA. CVs ranged from 4 to 13%, with most of them below 8%.

A dip-stick IA using mAb immobilized on a membrane was used for the determination of atrazine in water and liquid food samples.⁽⁴⁶⁾ The measuring range was $0.3\text{--}10\text{ }\mu\text{g L}^{-1}$ using reflectance detection. The total assay time was 25 min using precoated dip sticks. The atrazine concentrations could be determined directly in spiked water, milk and juice samples yielding satisfactory agreement with the spiking concentrations. The black tea samples, however, showed an overestimation due to the unspecific binding of the tannins to the membrane.

Paraquat was determined in milk, beef and potatoes using an EIA with pAb.⁽²³²⁾ Potatoes were shredded with dry ice. The potato and the meat samples were extracted with HCl after spiking the samples. The acid extracts were evaporated to dryness and reconstituted for the EIA. The milk was diluted with phosphate buffer. The EIA was able to detect less than 1 ppb of paraquat in whole milk and down to 2.5 ppb in beef. The efficiency of HCl for extracting potato and ground beef was determined by using methyl-¹⁴C paraquat. Recoveries between 60 and 70% were obtained. Since paraquat is known to bind tightly to many matrices, recoveries for both matrices were determined after storage of the spiked samples for several days at 21 °C.

Ibrahim et al.⁽²³³⁾ investigated eggs for aldrin and dieldrin residues. An EIA with pAb was applied. The EIA detected only dieldrin, but aldrin is metabolized to dieldrin. The eggs were homogenized after removal of the shell. This solution was diluted 1 : 2 with wash buffer containing 2% BSA. Egg samples were collected in Egypt and assayed by EIA. The standard curve for dieldrin was prepared in egg solution of noncontaminated eggs. The egg samples showed concentrations of aldrin and dieldrin in eggs up to 0.7 mg L^{-1} . These are concentrations above the World Health Organisation average daily intake levels.

5.4 Biomonitoring

Human biomonitoring involves the measurement of a parent chemical and/or metabolites or a product of its reaction with cellular components (e.g. protein adduct, nucleic acid adduct) in selected tissues, body fluids such as blood, milk, urine or sweat, or expired breath of an exposed individual.^(8,9) Most IAs for pesticides are sensitive enough for biomonitoring. Many analytes could be determined without any sample preparation other than dilution with water or buffer. In some cases the sample was filtered. Slightly reduced sensitivity or higher blank values due to matrix effects were sometimes found when

the assay was performed directly in the biological sample. With increasing hydrophobicity of the analyte, sample preparation becomes more complicated.⁽⁸⁾

Validation experiments are carried out by fortification of samples with the analyte in question and comparison with independent control methods such as GC, GC/MS or LC. Samples from a number of individuals should be used in any validation experiment because of the inherent variability of biological matrices. Features such as pH, protein, sugar and salt concentration must be considered. Also the intake of drugs can interfere with the immunological determination of environmental pollutants.⁽⁸⁾

While many investigations were restricted to spiked samples only, some studies demonstrated the use of IA for surveillance of occupationally exposed individuals (e.g. for DDA, a metabolite of DDT, 2,4-D, paraquat and atrazine mercapturate).^(140,171,234,235)

6 NEW DEVELOPMENTS

The strength of immunochemical methods lies in the screening of a large number of samples within a short time at low cost. Therefore, they can be valuable supplements to conventional analytical methods. Important applications are seen in the analysis of ground and drinking water, where matrix effects are seldom observed. Also, food commodities that turn over quickly are ideal targets for IA measurements. Owing to the low cost of one analysis more replicates from one site can be measured or special sites can be sampled more often, for example to obtain more information about variations of analyte concentrations depending on seasons or rainfall, etc.

Some restrictions are imposed by the fact that IAs are de facto single analyte methods. However, new approaches for multianalyte measurements are being undertaken, such as the integration of IA with LC. Here, Abs are used in conjunction with LC, e.g. to concentrate an analyte from a large volume of sample and separate it from an interfering matrix.^(236–239) In this case an immunoabsorbent column is used before analysis by LC. The immunoabsorbent column contains immobilized specific Abs which bind the analyte, while interfering substances pass through. The analyte can be eluted by using a pH gradient⁽⁷⁹⁾ or an organic solvent.⁽⁸⁰⁾ Therefore, large sample volumes with low concentrations of the analyte can be reduced to small volumes with sufficiently high concentrations without coextracting interfering substances like HAs or food compounds. This raises the effective sensitivity of the analysis. Ab mixtures can be used to bind substances from different compound classes, e.g. the phenyl urea herbicides and the triazines.⁽²³⁹⁾ In this case, the eluted compounds were

injected into the LC, yielding a DL of $0.03\text{--}0.5\mu\text{g L}^{-1}$ from sample volumes as low as 25 or 50 mL.

When cross-reacting Abs are applied in IA, the signal obtained is not only related to the analyte, but also to similar compounds. This problem can be circumvented by the use of LC prior to the IA. LC/IA was applied by Krämer et al.⁽²³⁶⁾ to determine 4-nitrophenols. The nitrophenols were separated by different LC systems and determined by IA. LC/IA was about 8–10 times more sensitive compared with LC with ultraviolet detection. Therefore, the integration of LC with IA combines the high separation quality of LC and the sensitivity of IA.^(240,241)

Furthermore, multianalyte systems are under development. One concept is the microspot IA,⁽²⁴²⁾ which uses many microspots with fluorescence-labeled Abs of different selectivity immobilized on a chip. After incubation with the analyte (Ag or hapten) a fluorescence-labeled tracer Ab is added. The tracer Ab is either directed against the Ag or consists of an anti-idiotypic Ab directed against the binding site of the capture Ab. Sensor and tracer Ab carry different fluorescence labels. Therefore it is possible to determine the amount of analyte bound to the sensor Abs with optical scanning methods by measuring the signal ratio (ratiometric assay). Lately, a variety of noncompetitive and competitive microspot analysis systems have been developed, mainly related to the medical field,⁽²⁴³⁾ but are clearly of particular importance in areas such as environmental monitoring.

Another possibility is the use of cross-reacting Abs for multianalyte detection. Known cross-reactivities of different Abs can be used to calculate the different concentrations of different analytes in a sample containing several contaminants.⁽²⁴⁴⁾ The estimation of the individual concentrations is carried out by complex calculating procedures, e.g. by neural networks⁽²⁴⁵⁾ or iterative procedures.⁽²⁴⁶⁾

Immunochemical analysis is a fast developing field with numerous possibilities for further improvement. Much effort is being put into the development of continuous measurements, such as flow-injection immunoanalysis (FIIA) and immunosensors.^(247,248) A quasicontinuous FIIA of pesticides was developed by Krämer and Schmid⁽²⁴⁹⁾ on the basis of a competitive IA. Here, the Abs are immobilized on a membrane. The reaction takes place in the membrane reactor, the central part of the flow injection system. All reagents are sequentially added to the reactor and the product is assayed with the aid of a flow fluorimeter. The measuring range of the flow injection analysis almost equals that of the EIA. Wittmann and Schmid⁽²⁵⁰⁾ used an Ab column reactor filled with polystyrene or glass beads with the Ab immobilized via the avidin/biotin system. This system showed a stable Ab activity for a minimum of 500

measuring cycles. DLs for atrazine of about 1 ng L^{-1} with pAb and 30 ng L^{-1} with mAb could be reached.

Important progress is to be expected in the field of immunosensors where the detectors are based on Abs.⁽²⁴⁷⁾ Some relatively simple devices such as dip sticks and immunofiltration assays have been mentioned before (see section 3.3). An interesting development is liposome-amplified immunomigration strips.^(251,252) They employ liposome-encapsulated markers which act as signal enhancers of the competitive binding reaction instead of enzymes. These devices have been used for the determination of alachlor.⁽²⁵¹⁾ If a pesticide of interest is conjugated to a lipid it can also be incorporated into the liposome structure, leading to a competitive liposome IA.⁽²⁵³⁾ In more complicated systems the immunological recognition system is immobilized in the direct vicinity of a transducer, an electrochemical, optical or gravimetric device. They respond to chemical compounds or ions and yield electrical signals which depend on the concentration of the analyte. Immunosensors with piezoelectric crystals as physical sensors are in a relatively advanced state of development.⁽²⁵⁴⁾ They function as microbalances onto which Abs are immobilized. Other physical sensors use optical systems such as surface plasmon resonance (SPR), interferometry or grating couplers.^(248,255,256) A biosensor employing SPR was used for the determination of atrazine.⁽²⁵⁶⁾ A DL of $0.05\mu\text{g L}^{-1}$ for atrazine in water was reached with an analysis time of 15 min. Bier and Schmid⁽²⁵⁷⁾ used a grating coupler immunosensor for the determination of terbutryn, a triazine herbicide. A DL of 15 nmol L^{-1} (ca. $3.6\mu\text{g L}^{-1}$) was established. Interesting developments can be expected from Ab electrodes.⁽²⁵⁸⁾

New strategies for Ab production are also being developed. Genetically engineered Abs appear very attractive because their selectivity and affinity can be tailored by site-directed mutations without requiring new immunizations.⁽²⁵⁹⁾ Methods are now provided to isolate desired clones rapidly from Ab libraries and to manipulate individual rAbs to match specific demands of environmental analysis. Binding proteins derived from Abs but consisting only of a part of their light or heavy chain (scFv, Figure 2) and rAb fragments, Fab, directed against different s-triazines, diuron and parathion have been produced.^(260–264) In several cases the DL of the rAb was the same as with the parent mAb.^(263,264)

A promising goal is the completely synthetic production of binding proteins or other synthetic receptors which are fitted to the structure of the analyte by molecular design. The use of libraries guarantees to close the bottleneck in Ab production. Abs with special properties such as resistance to matrix effects or organic solvent stability can also be selected from libraries, providing an

important contribution to the analysis of water and food samples.

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ABBREVIATIONS AND ACRONYMS

Ab	Antibody
Ag	Antigen
AOAC	Association of Official Analytical Chemists
BSA	Bovine Serum Albumin
CDR	Complementarity Determining Region
CLIA	Chemiluminescent Immunoassay
CV	Coefficients of Variation
DDA	Bis(<i>p</i> -chlorophenyl)acetic Acid
DDT	1,1,1-Trichloro-2,2-bis-(<i>p</i> -chlorophenyl)ethane
DL	Detection Limit
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
ESIMS	Electrospray Ionization Mass Spectrometry
Fab or F(ab) ₂	Antibody Fragments Containing the Antigen Binding Site(s)
Fc	Antibody Fragment Containing the Crystallizable Fragment
FIA	Fluorescence Immunoassay
FIIA	Flow-injection Immunoanalysis
FR	Frame Region
GC	Gas Chromatography
GC/LC	Gas Chromatography/Liquid Chromatography
GC/MS	Gas Chromatography/Mass Spectrometry
HA	Humic Acid
HPLC	High-performance Liquid Chromatography
IA	Immunoassay
IC ₅₀	Middle of Assay, Concentration of Analyte that Causes 50% Inhibition
Ig	Immunoglobulin
KLH	Keyhole Limpet Hemocyanin

LC	Liquid Chromatography
mAb	Monoclonal Antibody
MALDIMS	Matrix-assisted Ultraviolet Laser Desorption/Ionization Mass Spectrometry
MCPB	4-(4-Chloro-2-methylphenoxy)butyric Acid
pAb	Polyclonal Antibody
PCP	Pentachlorophenol
PFIA	Polarization Fluoroimmunoassay
rAb	Recombinant Antibody
RIA	Radioimmunoassay
scFv	Recombinant Antibody Fragment, Single Chain Fragment Containing only the Variable Region
SFE	Supercritical Fluid Extraction
SPE	Solid-phase Extraction
SPR	Surface Plasmon Resonance
2,4-D	2,4-Dichlorophenoxyacetic Acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic Acid

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